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What doesn't kill you doesn't make you stronger: Parasites modify interference competition between two invasive amphipods

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Abstract

We used a freshwater amphipod-microsporidian model (Ponto-Caspian hosts: *Dikerogammarus villosus* and *D. haemobaphes*, parasite: *Cucumispora dikerogammari*) to check whether parasites affect biological invasions by modulating behaviour and intra- and interspecific interactions between the invaders. We tested competition for shelter in conspecific and heterospecific male pairs (one or both individuals infected or non-infected). In general, amphipods of both species increased their shelter occupancy time when accompanied by infected rather than non-infected conspecifics and heterospecifics. Infected amphipods faced lower aggression from non-infected conspecifics. Moreover, *D. villosus* was more aggressive than *D. haemobaphes* and more aggressive towards conspecifics vs. heterospecifics. In summary, infection reduced the intra- and interspecific competitivity of amphipods, which became less capable of defending their shelters, despite their unchanged need for shelter occupancy. *Dikerogammarus haemobaphes*, commonly considered as a weaker competitor, displaced by *D. villosus* from co-occupied locations, was able to compete efficiently for the shelter with *D. villosus* when microsporidian infections appeared on the scene. This suggests that parasites may be important mediators of biological invasions, facilitating the existence of large intra- and interspecific assemblages of invasive alien amphipods.

Keywords

aggression, Amphipoda, biological invasions, coexistence, *Dikerogammarus*, interference competition, Microsporidia, parasitic infection

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Introduction

Animal behaviour is known to be modulated by parasites, simply by their pathogenicity and inducing defence responses in their hosts (Satinoff 2011; Żbikowska and Cichy 2012), but also by increasing parasites' fitness through host manipulation (Bakker et al. 1997; Lagrue et al. 2007; Flegr 2015). Parasite-induced changes are usually multidimensional, altering multiple phenotypic traits by a single parasite (Cezilly and Perrot-Minnot 2010; Cezilly et al. 2013). This includes such aspects as morphology (Bakker et al. 1997), habitat selection (Żbikowska and Cichy 2012; Rachalewski et al. 2018), mobility (Dezfuli et al. 2003), boldness (Flegr 2015), aggression (Thomas et al. 2005; Mikheev et al. 2010), foraging (Fielding et al. 2003; Bącela-Spychalska et al. 2014) and reproduction (Hall et al. 2007). These changes may further propagate to the impact on ecosystem functioning (Friesen et al. 2017; Anaya-Rojas et al. 2019) through intra- and interspecific interactions of infected individuals with other organisms through consumption (Fielding et al. 2003), predation (Bakker et al. 1997; Flegr 2015; Friesen et al. 2019) or competition (Anderson and May 1986; Mikheev et al. 2010; Reisinger et al. 2015; Friesen et al. 2018).

Through these mechanisms, parasites may indirectly affect the process of biological invasions (Hatcher et al. 2015), which are considered as one of the most important threats to global biodiversity (Lambertini et al. 2011). Knowledge of ecology of invasive alien species, including their interactions with parasites, is crucial to understand the functioning of ecosystems in the present world (Dunn 2009; Dunn et al. 2012; Roy et al. 2016). Alien species in their novel areas may "escape" from their sympatric parasites and, in accordance with the enemy release hypothesis, get advantage over local biota, suffering standard levels of parasite infestation (Colautti et al. 2004; Dunn 2009; Heger and Jeschke 2014). On the other hand, parasites, both originating from the native range and locally acquired, may limit the spread of the alien hosts (Bojko et al. 2018; Chalkowski et al. 2018) by reducing their competitive ability. A more subtle influence of parasites may consist of modifications of the impact imposed by alien species on local communities (Dunn 2009) by changing their behaviour, e.g. food acquisition or preferences (Bącela-Spychalska et al. 2014; Iltis et al. 2017).

Alien species interfere not only with the local biota, but also with one another as competitors (Dick and Platvoet 2000), prey/predators (Borza et al. 2009), habitat engineers providing shelters (e.g. mussels, Kobak et al. 2009) and sources of interspecific semiochemicals (Rachalewski et al. 2019). The outcome of these interactions can be coexistence, displacement or facilitation. Facilitation, if prevailing at the community level, can contribute to the phenomenon of invasional meltdown (Simberloff and von Holle 1999). Parasites may mediate these interactions and affect their outcomes.

A perfect model to study multi-species interactions among invasive alien species and their parasites is the freshwater assemblage of Ponto-Caspian amphipod crustaceans and their intracellular microsporidian parasites (Bojko and Ovcharenko 2019). These amphipods interfere with invaded European environments as predators of invertebrate fauna (Krisp and Maier 2005), shredders (Truhlar et al. 2013), food sources

for fish (Grabowska and Grabowski 2005; Borza et al. 2009) and competitors of their local relatives (Dick et al. 2002). They occupy similar ecological niches (Dedju 1980), which makes them natural competitors among themselves (e.g. van Riel et al. 2007; Platvoet et al. 2009b; Jermacz et al. 2015), but they can also prey on one another (intra-guild predation) (Dick and Platvoet 2000; Kinzler et al. 2009) and communicate interspecifically, e.g. perceiving heterospecific alarm cues (Rachalewski et al. 2019). In their native region, these amphipods are infected with several species of Microsporidia (Wattier et al. 2007; Ovcharenko et al. 2009, 2010; Bącela-Spychalska et al. 2018), which hitch-hiked with their hosts to novel regions in Europe (Wattier et al. 2007; Ovcharenko et al. 2010; Bącela-Spychalska et al. 2012, 2018; Grabner et al. 2015). These parasites may reach high prevalence, up to 72% (Ovcharenko et al. 2010; Bojko et al. 2015; Iltis et al. 2017) and high ecological importance. Some of them can affect their host's behaviour, such as activity and predation (Bacela-Spychalska et al. 2014; Farahani et al. 2021). The strength of parasite impact depends on the transmission mode: horizontal, vertical or both (Dunn et al. 2001; Bącela-Spychalska et al. 2014; Bojko et al. 2018; Rachalewski et al. 2018). In general, the horizontal transmission is often linked to high virulence (Fielding et al. 2005), while vertical transmission is associated with low or no virulence, or even with the increased host fitness (e.g. Slothouber Galbreath et al. 2004). Moreover, Microsporidia with horizontal transmission can increase female-biased sex ratio, either by feminization or male killing (Kelly et al. 2002; Terry et al. 2004; Green-Etxabe et al. 2015).

To study the potential impact of microsporidiosis on the behaviour and mutual interactions among the Ponto-Caspian amphipods, we focused on two model species: *Dikerogammarus villosus* (Sowinsky, 1894) and *Dikerogammarus haemobaphes* (Eichwald, 1841), considered as successful invasive alien species in Europe (Rewicz et al. 2015; Jażdżewska et al. 2020). They are widespread across Europe and co-occur in many places, sharing similar habitat preferences and life history traits (Grabowski et al. 2007; Bovy et al. 2015; Clinton et al. 2018). Usually *D. villosus* dominates and displaces *D. haemobaphes* (Kley and Mayer 2003; Bollache et al. 2004; Gruszka and Woźniczka 2008; Kinzler et al. 2009; Žganec et al. 2009; Bącela-Spychalska et al. 2012), though opposite situations have also been reported from the UK, where *D. haemobaphes* is more widely distributed (Clinton et al. 2018).

Several microsporidian parasites were identified to often infect these two model host species, both in native and colonised ranges: *Cucumispora dikerogammari* (Ovcharenko et al. 2010; Bącela-Spychalska et al. 2012, 2014), *Dictyocoela duebenum*, *D. berillonum*, *D. muelleri* (Wattier et al. 2007; Grabner et al. 2015; Green-Etxabe et al. 2015; Bącela-Spychalska et al. 2018) and *C. ornata* recorded from *D. haemobaphes* (Bojko et al. 2015, 2018). Some more Microsporidia are known to infect these hosts only from single records, e.g. *Nosema granulosis* (Wattier et al. 2007), thus these can be considered as accidental infections. These parasites differ in their impact on hosts, however such data are not available for all the species. *Cucumispora dikerogammari*, infecting both model *Dikerogammarus* species, has successfully spread to most European waters with its hosts (Wattier et al. 2007; Ovcharenko et al. 2010). This parasite, having a direct

life cycle, is considered as a highly virulent parasite, transmitted mostly horizontally through consumption and causing behavioural changes and mortality (Ovcharenko et al. 2010; Bącela-Spychalska et al. 2012, 2014). Nevertheless, the effect of parasitic Microsporidia on the functioning of amphipod assemblages has been still understudied.

We tested experimentally how the presence of parasitic *Cucumispora dikerogammari* modulates shelter competition between the two invasive amphipod species. We hypothesized that: (1) Amphipod behaviour would depend on (a) species (irrespectively of infection status) and (b) infection status; (2) Infection would affect intraspecific interactions among amphipods by weakening infected conspecifics (as being in a worse physical condition); (3) Non-infected individuals would avoid aggression towards and/or contacts with infected specimens to reduce the risk of infection, as the parasite is mainly horizontally transmitted, through biting or consumption of infected tissue (Ovcharenko et al. 2010); (4) Amphipods would respond differently to conspecifics and heterospecifics; (5) Infection would affect interspecific interactions among amphipods by reducing the impact of infected individuals on their opponents (due to the mechanisms postulated in H2–3).

Materials and methods

Test organisms

We sampled *D. villosus* using artificial substratum traps in the Włocławek Dam Reservoir located in the lower Vistula River (N52.617738, E19.326453) and *D. haemobaphes* with benthic hand nets in the middle part of the Vistula River near the town of Połaniec (N50.423014, E21.311748) during the last week of May 2018. We transported the animals to the laboratory in plastic buckets with aerated water, placed in Styrofoam boxes filled with ice packs. We kept them in plastic containers (40 × 60 × 12.5 cm, L × W × H) with gravel substratum (grain size 2–5 cm) at their average natural densities (c.a. 400 ind. m^{-2}) (Dedju 1967). We used conditioned tap water, air-conditioning (17 °C) and light:dark cycle of 16:8 (which corresponded to the conditions at the sampling sites), and fed the amphipods daily with living chironomid larvae (commercially purchased) and dry fish food pellets. Every 3 days, we exchanged 30% of water. After one week of acclimatization, we used them in experiments. To avoid potential differences in aggression level between sexes as well as reproductive rather than aggressive relationships in conspecific pairs, we used only males in our experiments, distinguishing them by the presence of dense and long setation on the flagellum of the antenna II and large gnathopods (Eggers and Martens 2001). For each individual, we identified the species before the experiment by morphological features, such as the setation of the flagellum of the antenna II and the shape of the dorsal tubercles on the urosome segments I and II (Eggers and Martens 2001; Konopacka 2004). These two features are easy to observe and allow to discriminate between males of the two species by eye.

The very late stage of microsporidiosis is manifested by the whitish colour of the infected tissue (muscles), visible through the host cuticle even by eye (Ovcharenko et al. 2010). Symptomatic individuals can be in the lethal phase, likely to die in a few days (Bącela-Spychalska et al. 2012). Therefore, in the experiments, we used infected individuals before they started to exhibit any external symptoms of infection. Hence, we were able to detect and identify microsporidian infections only using molecular methods possible to apply after the experiments (see "Detection and identification of microsporidian parasites"). For the experiment, we used animals with natural infections acquired in the field, which reflected the situation in the environment. After the molecular diagnosis, we determined that *D. villosus* had been infected by a single Microsporidium species: *C. dikerogammari*, whereas *D. haemobaphes* was the host for three species: *C. dikerogammari*, *Dictyocoela berillonum* and *D. muelleri*. Due to insufficient numbers of all pairwise combinations of infection types, we only used non-infected amphipods and those infected with *C. dikerogammari* for our study to obtain a balanced design and sufficient number of replicates (Suppl. material 1: Table S1).

Experimental protocol

We performed experiments in glass dishes (diameter: 90 mm, height: 45 mm). A 20-mm high Plexiglas disk of the same diameter as the dish was put on its bottom. A hole (diameter: 7 mm, depth: 17 mm) was drilled in the disk 3 mm from its edge (Suppl. material 1: Fig. S1) to form a single shelter for amphipods. Such shelters were evidenced as suitable for amphipods, including *D. villosus*, by Platvoet et al. (2009b). We sealed gaps between the disk edges and glass walls of the dish with white plasticine to prevent amphipods from entering this space. The plasticine was proven as non-toxic for amphipods in our preliminary trials.

We aimed at testing shelter competition in all possible species vs. infection status combinations. We preliminarily screened both amphipod populations for the prevalence of various microsporidian species (see: "Detection and identification of microsporidian parasites" section), based on 100 individuals of each host species. This allowed us to roughly estimate the number of pairs to be tested to obtain sufficient numbers of all combinations. Altogether, we tested 80 conspecific pairs of *D. haemobaphes*, 219 conspecific pairs of *D. villosus* and 254 heterospecific pairs (Suppl. material 1: Table S1). We selected male individuals randomly with regard to their size.

We placed a pair of amphipods, both individuals marked with correction fluid to identify them during the analysis (the fluid and the marking procedure were proven as harmless during our preliminary trials), into an experimental dish, allowed them for 5-min acclimatization and recorded their behaviour for the next 30 min using a video camera (SNB-6004, Samsung, South Korea) located above the experimental arena. Water temperature was the same as in the stock tanks. Water was oxygenated before the test, thus, given its short duration, we assume that oxygen was not a limiting factor for the amphipods. After the test, we dried amphipods with a paper towel for 30 s to get rid of excess water (as described by Pöckl 1992) and weighed them to the nearest

0.01 g using a Kern microbalance (type PEJ, Germany) (wet weight) to assess their size. Finally, after the experiment, we fixed them individually in 96% ethanol to conduct molecular screening for the microsporidian presence and identification.

After molecular determination of microsporidian presence in each individual (see: "Detection and identification of microsporidian parasites" section), processed after the experimental trials, we were able to assign particular previously tested amphipod pairs to specific experimental treatments regarding their infection status (see Suppl. material 1: Table S1 for details).

Detection and identification of microsporidian parasites

We dissected muscle tissues from individual amphipods stored in 96% ethanol with forceps and incubated them at 55 °C in 1.5-ml tubes containing 200 µl of Queen's lysis buffer with 5 µl of proteinase K (20 mg ml^{-1}) according to the procedure by Seutin et al. (1991). We extracted total DNA (including microsporidian DNA, if present) using the standard phenol/chloroform method by Hillis et al. (1996) and resuspended dried DNA in 100 µl of TE buffer at pH 8 and stored at 4 °C until amplification. We conducted the PCR and used a pair of microsporidia-specific primers V1f/530r, following Baker et al. (1994) and Vossbrinck et al. (1993) to amplify distinctive parasite DNA fragments. We ran PCR reactions in 10 µl of reaction mixtures with each primer concentration of 400 nM, 200 µM dNTPs and 0.5 U/µl Taq polymerase (Thermo Scientific). The product was amplified under the following PCR conditions: an initial denaturing step at 95 °C for 2 min was followed by 35 cycles of 95 °C for 30 s, 62 °C for 45 s and 72 °C for 1 min. These cycles were followed by a final extension at 72 °C for 5 min. We included a negative control containing no DNA and a positive control containing the known Microsporidium species in each set of PCR reactions. We visualised The PCR product on 2% agarose gel in order to identify positives - the presence of microsporidian DNA. Afterwards, we purified the selected positives with exonuclease I (Burlington, Canada) and FastAP alkaline phosphatase (Fermentas, Waltham, MA, USA) treatment and sequenced them directly with the BigDye technology by Macrogen Inc., (Amsterdam, The Netherlands) using the above-mentioned primers. We edited the obtained microsporidian sequences using Geneious R10 (http://www.geneious.com, Kearse et al. 2012). Then, we conducted the identification of microsporidia using BLAST in GenBank (https://www.ncbi.nlm.nih.gov/genbank).

Data analysis

We watched all the video recordings of amphipod behaviour manually (always the same person, to avoid bias) to determine: (1) Time spent in shelter by each individual, (2) Counts of aggression acts exhibited by each individual, when an amphipod touched the other individual with its antennae I and attempted to catch it with its gnathopods and antennae II (described as a sign of aggression by Platvoet et al. 2009b),

and (3) Time spent together in shelter by both individuals from the pair. Time variables were expressed as percentages of the total experimental time.

To test our hypotheses, we conducted four sets of General Linear Models (for time-related variables) and Generalized Linear Models with Poisson distribution and log link function (for aggression counts) using various subsets of the entire dataset (summarised in Suppl. material 1: Table S2). The division of the dataset was necessary due to the nature of the data, as explained below. The full dataset with subsets used for particular analyses described below is available in Suppl. material 2.

(1) To analyse intraspecific relationships among amphipods, we tested non-infected and infected individuals accompanied by non-infected and infected conspecifics. We had to divide this analysis into four separate models (Suppl. material 1: Table S2A-D), as the dataset followed partly a within-subject design (two members of a mixed pair consisting of an infected and non-infected individual were exposed together) and partly a between-subject design (selected members of uniformly infected and non-infected pairs were exposed in separate runs). Thus, to check the effect of the animals' own infection on their responses to conspecifics, we tested separately: (i) non-infected and infected responding amphipods accompanied by non-infected conspecifics (Suppl. material 1: Table S2A) and (ii) non-infected and infected responding amphipods accompanied by infected conspecifics (Suppl. material 1: Table S2B), using models including: (i) species and (ii) infection status of the responding individual. Moreover, to check the effect of the accompanying conspecific infection, we tested separately: (i) non-infected responding amphipods accompanied by non-infected or infected conspecifics (Suppl. material 1: Table S2C) and (ii) infected responding amphipods accompanied by non-infected or infected conspecifics (Suppl. material 1: Table S2D), using models including: (i) species and (ii) infection status of the accompanying individual.

(2) To check whether amphipods responded differently to individuals of various species, we compared the behaviour of non-infected and infected amphipods in the presence of conspecifics and heterospecifics (for simplification: non-infected only). Separate models were conducted for each amphipod species (Suppl. material 1: Table S2E-F), as conspecific/heterospecific identity was not the same for *D. villosus* and *D. haemobaphes*. The models included (i) infection status of the responding individual and (ii) accompanying species (conspecific or heterospecific).

(3) To check the effect of infection status on interspecific interactions among amphipods, we tested heterospecific pairs differing in infection status. The model (Suppl. material 1: Table S2G) included: (i) species of the responding individual (a within-subject factor, as both individuals of a heterospecific pair were included), (ii) infection status of the responding individual and (iii) infection status of the accompanying individual.

Using the above-mentioned models 1–3, we tested two response variables: time spent by responding amphipods in shelter and number of their aggression acts. Moreover, to further check the effect of infection status on intra- and interspecific interactions among amphipods we compared:

(4) The time spent together in the shelter by both individuals of the pair, using a model (Suppl. material 1: Table S2H) with treatment effect (all available combinations of species and infection status).

We selected responding animals from uniform pairs (conspecifics of the same infection status) randomly for the analyses. To control for the difference between masses of pair members, likely to affect the competition, we included a mass ratio (responding/accompanying individual) as a continuous predictor in models 1–2 above. In model 3, we included individual masses of both amphipods from each heterospecific pair as a continuous predictor. In model 4, we controlled for the effect of mass by including a mass ratio (larger/smaller individual) as a continuous predictor. We log-transformed the time-related variables prior to the analysis to meet General Linear Model conditions (normality tested with a Shapiro-Wilk test, homoscedasticity tested with a Levene test). As needed (i.e. when significant effects had more than 2 levels), we used sequential Bonferroni corrected pairwise LSD Fisher tests (General Linear Models) or pairwise contrasts (Generalized Linear Models) for post-hoc comparisons. We conducted all statistical analyses using SPSS 27.0 statistical package (IBM Inc.).

Results

Intraspecific interactions among amphipods

Differences between the species. The only interspecific difference in shelter occupancy was the longer time spent in shelter by *D. villosus* compared to *D. haemobaphes* exposed to non-infected conspecifics (Fig. 1A, a significant species effect in Table 1A). Moreover, intraspecific aggression of *D. villosus* was always higher than that of *D. haemobaphes* (Fig. 1B, significant species effects in Table 1A–D).

Effect of the infection status of the responding individual. The infection status did not affect time spent by amphipods in shelter and their aggression in the presence of non-infected conspecifics (non-significant infection effects for both behaviours in Table 1A). On the other hand, in the presence of infected conspecifics, non-infected amphipods of both species occupied shelters for a longer time (Fig. 1A) and were less aggressive (Fig. 1B) than infected individuals (significant infection effects for both behaviours in Table 1B).

Effect of the infection status of the accompanying conspecific. Individuals of both species, irrespective of their own infection status, spent more time in shelter in the presence of infected rather than non-infected conspecifics (Fig. 1A), as shown by significant infection effects in Table 1C, D). Non-infected amphipods of both species were more aggressive towards non-infected than towards infected conspecifics (Fig. 1B, a significant infection effect in Table1C), whereas intraspecific aggression of infected amphipods was unrelated to the infection status of accompanying conspecifics (Fig. 1B, Table 1D).

Table 1. Analyses of the effect of infection and species identity on intra- and interspecific interactions among amphipods. We analysed shelter occupancy time and number of aggression acts with the General and Generalized Linear Models (Poisson distribution, log link function), respectively.

Analysis	Effect	*df*	Time in shelter		Aggression	
			F	*P*	*F*	*P*
A. Responses of infected vs. non-infected amphipods to non-infected conspecifics	Species1	1, 210	6.63	0.011*	76.23	<0.001*
	Infection1	1, 210	2.50	0.115	0.01	0.935
	Sp1*Inf1	1, 210	2.53	0.113	0.03	0.855
	Mass ratio	1, 210	0.25	0.618	5.71	0.018*
B. Responses of infected vs. non-infected amphipods to infected conspecifics	Species1	1, 177	0.66	0.417	17.94	<0.001*
	Infection1	1, 177	8.66	0.004*	23.21	<0.001*
	Sp1*Inf1	1, 177	0.25	0.620	1.50	0.222
	Mass ratio	1, 177	11.21	0.001	6.59	0.011*
C. Responses of non-infected amphipods to infected vs. non-infected conspecifics	Species1	1, 210	0.16	0.692	19.79	<0.001*
	Infection2	1, 210	19.00	<0.001*	14.63	<0.001*
	Sp1*Inf2	1, 210	1.50	0.223	0.03	0.865
	Mass ratio	1, 210	3.97	0.048	7.95	0.005*
D. Responses of infected amphipods to infected vs. non-infected conspecifics	Species1	1, 177	1.99	0.160	39.39	<0.001*
	Infection2	1, 177	5.40	0.021*	0.02	0.890
	Sp1*Inf2	1, 177	2.91	0.090	3.50	0.063
	Mass ratio	1, 177	3.31	0.070	1.64	0.202
E. Responses of infected vs. non-infected *D. haemobaphes* to non-infected conspecifics vs. heterospecifics	Species2	1, 338	1.80	0.181	0.17	0.677
	Infection1	1, 338	1.63	0.202	1.54	0.215
	Sp2*Inf1	1, 338	1.08	0.300	0.65	0.420
	Mass ratio	1, 338	0.12	0.728	19.31	<0.001*
F. Responses of infected vs. non-infected *D. villosus* to non-infected conspecifics vs. heterospecifics	Species2	1, 338	0.44	0.510	32.28	<0.001*
	Infection1	1, 338	1.32	0.251	2.85	0.092
	Sp2*Inf1	1, 338	1.33	0.249	1.98	0.160
	Mass ratio	1, 338	4.99	0.026	4.70	0.031*
G. Responses of infected vs. non-infected amphipods to infected vs. non-infected heterospecifics	Species1[WS]	1, 499	4.55	0.033*	5.48	0.020*
	Infection1	1, 499	1.37	0.243	2.19	0.140
	Infection2	1, 499	0.86	0.356	0.05	0.830
	Sp1*Inf1	1, 499	1.92	0.166	0.04	0.847
	Sp1*Inf2	1, 499	5.91	0.015*	0.07	0.792
	Inf1*Inf2	1, 499	0.09	0.761	0.11	0.744
	Sp1*Inf1*Inf2	1, 499	0.09	0.771	0.21	0.650
	Mass	1, 499	0.46	0.498	3.62	0.058
H. Time spent together in the shelter	Pair comp.	9, 542	4.25	<0.001*		
	Mass ratio	1, 542	0.45	0.504		

Species1/2 (Sp1/Sp2) – species of the responding / accompanying individual, respectively (*D. haemobaphes* or *D. villosus*)
Infection1/2 (Inf1/Inf2) – infection status of the responding / accompanying individual, respectively (infected or non-infected)
Pair comp. – species composition and infection status of the amphipod pair in analysis H: both *D. haemobaphes*, both *D. villosus* or heterospecific × both infected, infected & non-infected or both non-infected
Mass ratio – responding / accompanying individual (analyses A-F) or larger / smaller individual (analysis H)
Mass – individual mass (analysis G)
WS – within-subject factor (analysis G)
Responding individuals from uniform pairs (conspecifics with the same infection status) in analyses A–F were randomly selected from the pair

Differences between intra- and interspecific interactions

Dikerogammarus haemobaphes did not change its shelter occupancy time and aggression depending on the species identity of the accompanying individual (Fig. 2, non-significant species effects for both behaviours in Table 1E). On the other hand, *D. villosus* was more aggressive towards conspecifics than towards *D. haemobaphes* (Fig. 2B, Table 1F), though its shelter occupancy time was unaffected by the accompanying species identity (Table 1F).

Interspecific interactions among amphipods

In the presence of *D. villosus*, *D. haemobaphes* spent more time in shelter when the accompanying individual was infected rather than non-infected (Fig. 3A). On the other hand, shelter occupancy time of *D. villosus* did not depend on the infection status of the accompanying *D. haemobaphes*, which resulted in a significant species*accompanying individual infection interaction (Table 1G). Shelter occupancy time by amphipods was independent of the infection status of the responding individual (non-significant effects involving the responding individual infection in Table 1G).

Interspecific aggression of *D. villosus* was higher than that of *D. haemobaphes* (Fig. 3B, a significant species effect in Table 1G), irrespective of the infection status of responding or accompanying individuals (non-significant infection effects in Table 1G).

Time spent by amphipod pairs together in shelter

Time spent together by both individuals in shelter depended on pair composition (Table 1H). Non-infected heterospecific pairs spent more time together in shelter than non-infected conspecific pairs (Fig. 4). Moreover, infected heterospecific and *D. villosus* pairs spent more time together in shelter than infected *D. haemobaphes* pairs. Furthermore, non-infected *D. villosus* pairs spent less time together in shelter than *D. villosus* pairs with at least one infected individual (Fig. 4).

Discussion

Interspecific differences

As predicted by hypothesis 1a, both species differed from each other in behaviour. Interspecific differences in shelter occupancy time were inconsistent. *Dikerogammarus haemobaphes* spent more time in the shelter than *D. villosus* when exposed to infected heterospecifics (Fig. 3A), whereas an opposite difference occurred between individuals of these species exposed to non-infected conspecifics (Fig. 1A). This suggests that shelter occupancy depended more on the identity of the accompanying individual (both species spent more time in shelter in the presence of accompanying *D. villosus*) than on the responding amphipod. Nevertheless, both intra- and interspecific aggression (Fig. 1B and Fig. 3B, respectively) of *D. villosus* was consistently higher than that of *D. haemobaphes*. The former species is often considered as the strongest competitor of all invasive Ponto-Caspian amphipods, aggressively displacing native and alien relatives (Dick and Platvoet 2000; Krisp and Maier 2005), including *D. haemobaphes* (Kley and Maier 2003; Žganec et al. 2009), from novel areas. Its high aggression level, enabling efficient interference competition, is congruent with high invasiveness (van Riel et al. 2009; Bertelsmeier et al. 2015; Grether et al. 2017). Both these species are

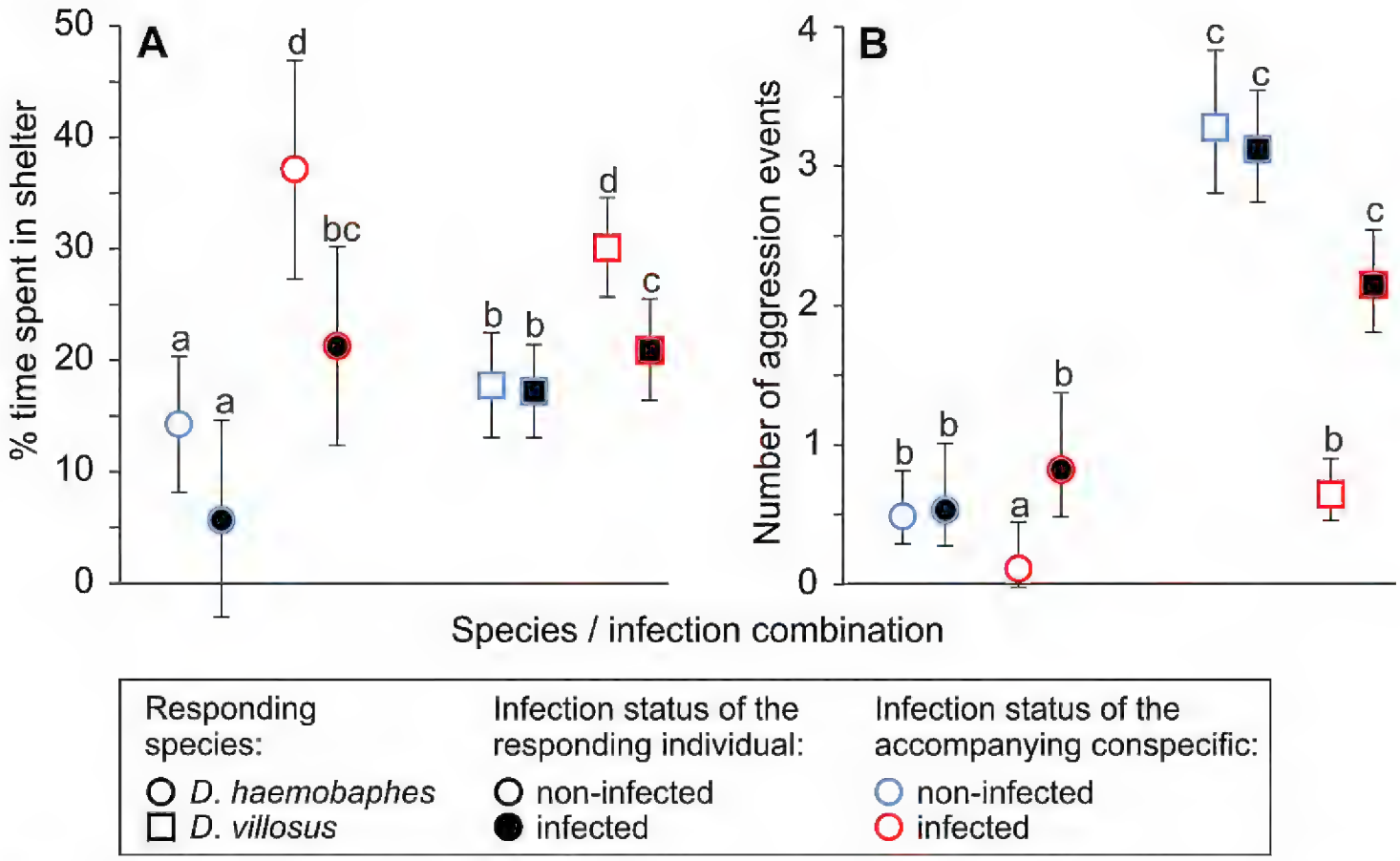

Figure 1. Effect of infection on intraspecific interactions among amphipods. Shelter occupancy time **A** and number of aggression acts **B** shown by infected (black) or non-infected (white) *D. haemobaphes* (circles) and *D. villosus* (squares) in response to infected (red border) or non-infected (blue border) conspecifics. Results are back-transformed least squares means (±95% confidence intervals) predicted for significant effects by the General or Generalized Linear Models (analyses A–D in Table 1 and Suppl. material 1: Table S2). Treatments marked with the same lowercase letters did not differ significantly from one another.

typical "sit and wait" organisms exhibiting low activity and spread rate (Platvoet et al. 2009a; Beggel et al. 2016), which is especially true for *D. haemobaphes* not exposed to interspecific competition (Kobak et al. 2016; Rachalewski et al. 2019).

Intraspecific interactions among amphipods

In conspecific pairs, the highest shelter occupancy time was exhibited by non-infected amphipods exposed to infected conspecifics (Fig. 1A). Thus the infection of the accompanying individual resulted in the higher shelter occupancy time, especially when the responding individual was not infected. Moreover, non-infected amphipods were less aggressive towards infected vs. non-infected conspecifics, confirming our hypothesis 3, whereas no such difference was exhibited by infected animals (Fig. 1B).

The increased shelter occupancy time in the presence of infected conspecifics indicates that infected individuals posed a lower competitive pressure. They were either more easily displaced from the shelter or allowed their competitors to occupy the shelter together with them. The fact that the amount of time spent together by both *D. villosus* individuals in the shelter increased when at least one of them was infected (Fig. 4) supports the latter explanation. The aggression of infected individuals was not reduced

(Fig. 1B), thus it is likely that they were less efficient in their attempts to seize the shelter than non-infected conspecifics, e.g. due to weaker condition or locomotor ability.

The reduced aggression of non-infected amphipods towards infected vs. infected conspecifics (Fig. 1B), in line with unchanged aggression level of infected individuals, suggest that the reduction in intraspecific aggression depended on the infection status of the attacked individual, rather than on that of the attacker. In other words, amphipods avoided to attack infected conspecifics (though did not avoid their company in the shelter). This may be accounted for by an attempt to reduce the probability of infection. *Cucumispora dikerogammari* is transmitted horizontally (by consumption, thus biting may be dangerous) and causes a lethal disease in their hosts (Bącela-Spychalska et al. 2012). Recognition of infected conspecifics and refraining from dangerous contacts with them is a widespread mechanism of infection avoidance in animals (Curtis 2014; Øverli and Johansen 2019). This shows that the effects of parasites on their hosts may be sometimes quite subtle, not manifested by direct changes in survival or appearance, but exhibited in specific situations, such as the high competitive pressure (MacNeil et al. 2003).

On the other hand, infected amphipods of both species did not diversify their responses depending on the infection status of their opponent (Fig. 1B). Thus, infection is likely to disrupt natural behaviour of amphipods, which may reduce their ability to respond appropriately to environmental factors.

To summarize, in accordance with our hypothesis 2, Microsporidia reduced competitive abilities of both amphipod hosts: infected individuals performed worse in shelter competition against their non-infected conspecifics.

Interspecific interactions among amphipods

Amphipod shelter occupancy time did not depend on the accompanying species identity (Fig. 2A), but, in accordance with our hypothesis 4, *D. villosus* was more aggressive towards conspecifics than towards *D. haemobaphes* (Fig. 2B). In contrast, the intra- and interspecific aggression levels of *D. haemobaphes* were similar to each other (Fig. 2B) and consistently lower than those of *D. villosus* (Fig. 3B). On the other hand, heterospecific pairs spent more time together in shelter than conspecific pairs of both amphipod species, suggesting the higher level of negative intraspecific relationships also in *D. haemobaphes* (Fig. 4). The higher intraspecific aggression shown by *D. villosus* in our study, is a common situation in the nature (Connell 1983). Conspecifics use the same resources and therefore pose a stronger competitive pressure than heterospecifics, even from the same guild. On the other hand, strong interspecific aggression was also indirectly observed in another pair of freshwater Ponto-Caspian amphipods, *D. villosus* and *Pontogammarus robustoides* (Jermacz et al. 2015) and many examples of strong interspecific interferences can be found in nature (Amarasekare 2002).

Surprisingly, *D. villosus* did not affect the shelter occupancy of *D. haemobaphes* more than conspecifics did (Fig. 2A). *Dikerogammarus haemobaphes* showed relatively low level of aggression towards both species. When given such a possibility, it usually migrates away from sites occupied by *D. villosus* (Kobak et al. 2016) and actively escapes from its scent (Rachalewski et al. 2019). Thus, *D. haemobaphes* seems to avoid direct encounters

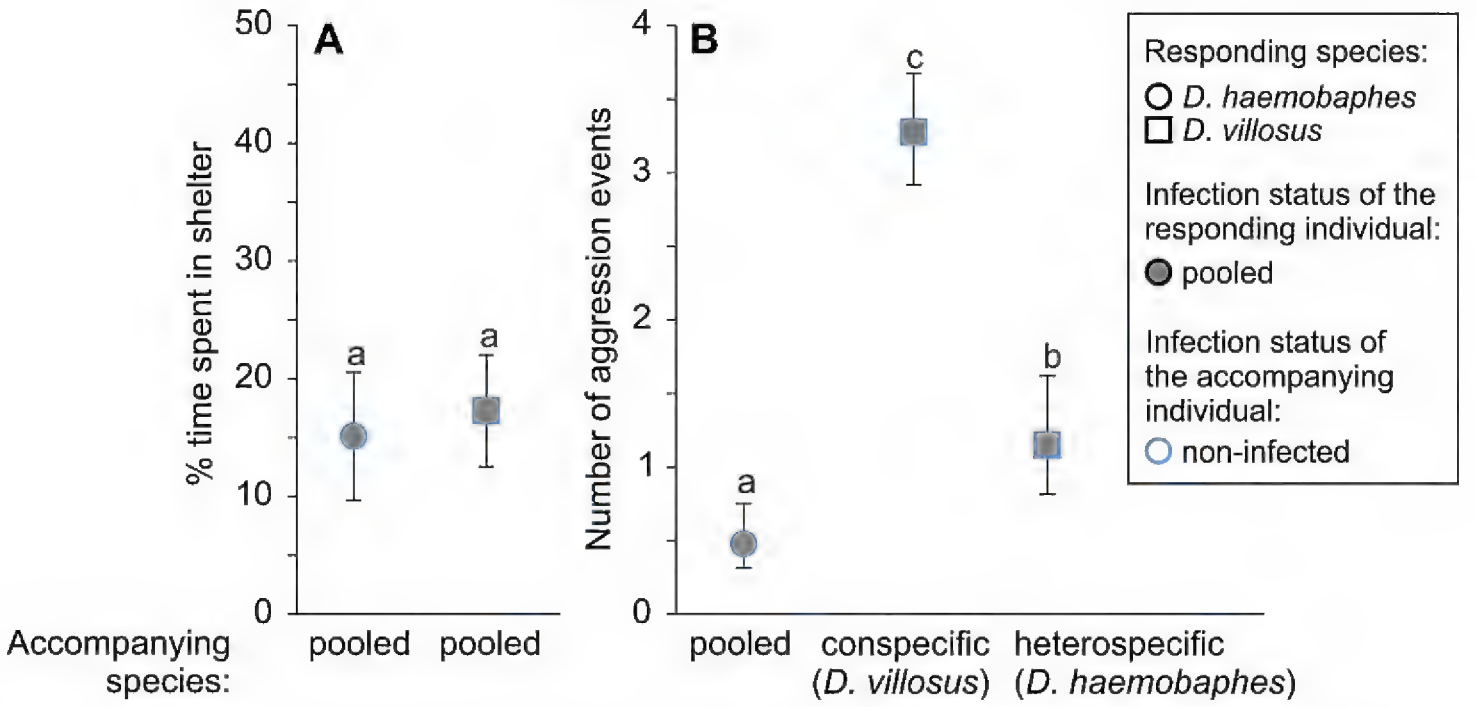

Figure 2. Amphipod responses to conspecific and heterospecific opponents **A** shelter occupancy time and **B** number of aggression acts shown by *D. haemobaphes* (circles) and *D. villosus* (squares) (pooled infection status) in response to non-infected conspecifics and heterospecifics. Results are back-transformed least squares means (±95% confidence intervals) predicted for significant effects by the General or Generalized Linear Models (analyses E-F in Table 1 and Suppl. material 1, Table S2). Treatments marked with the same lowercase letters did not differ significantly from one another. Non-significant effects are pooled.

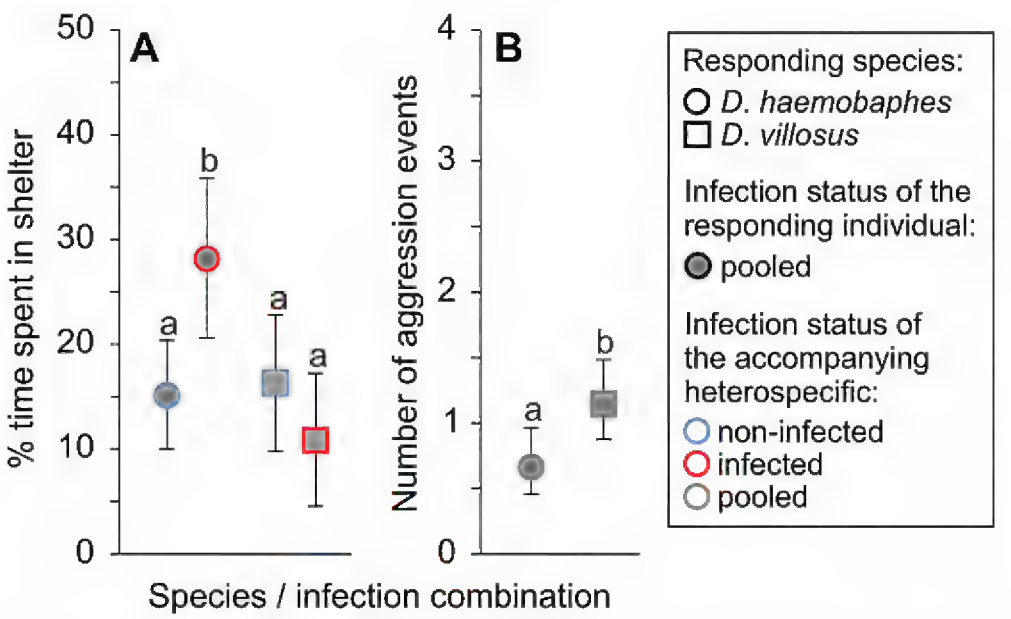

Figure 3. Effect of infection on interspecific interactions among amphipods **A** shelter occupancy time and **B** number of aggression acts shown by *D. haemobaphes* (circles) and *D. villosus* (squares) (pooled infection status) in response to heterospecifics of various infection status: infected (red border), non-infected (blue border) or pooled (grey border). Results are back-transformed least squares means (±95% confidence intervals) predicted for significant effects by the General or Generalized Linear Model (analysis G in Table 1 and Suppl. material 1, Table S2). Treatments marked with the same lowercase letters did not differ significantly from one another. Non-significant effects are pooled.

with *D. villosus* in the environment. That is why *D. villosus* might show lower aggression towards *D. haemobaphes* than towards conspecifics. The displacement between the two species, often observed in the field (Kley and Maier 2003), may depend on the active avoidance of *D. villosus* by *D. haemobaphes* rather than on direct aggression and interfer-

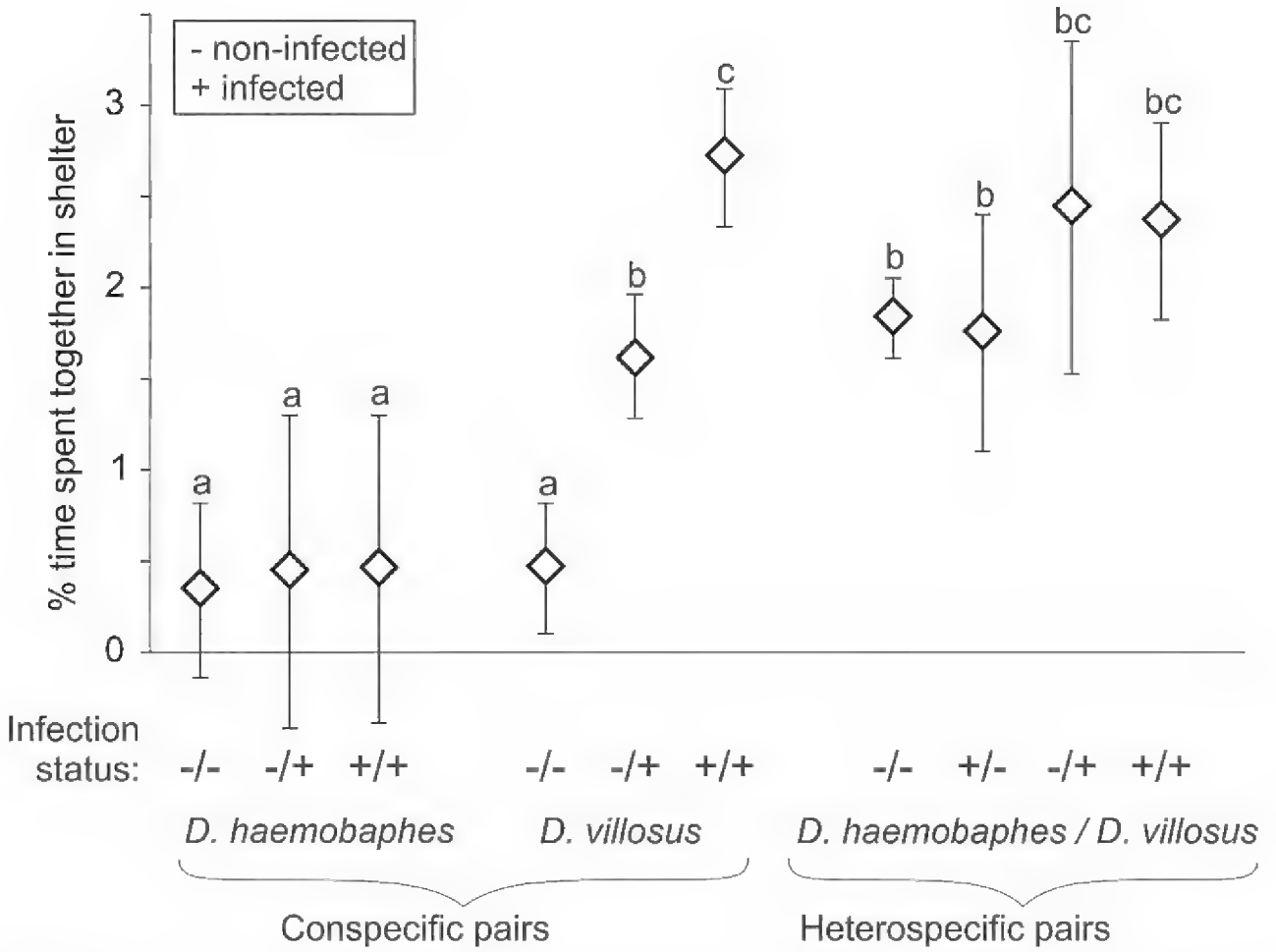

Figure 4. The effect of infection on time spent together in shelter by two amphipods. Pairs were composed of infected or non-infected *D. haemobaphes* and/or *D. villosus*. Results are back-transformed least squares means (±95% confidence intervals) predicted by the General Linear Model (analysis H in Table 1 and Suppl. material 1: Table S2). Treatments marked with the same lowercase letters did not differ significantly from one another.

ence competition among them. The ability to assess their own chances and avoid a direct conflict with a stronger opponent allows animals to minimize their energy losses and risk of injures (Parker and Rubenstein 1981). In the wild, *D. haemobaphes* can retreat to habitats avoided by its stronger competitor, e.g. with stronger water flow (Borza et al. 2018). Anyway, our study shows that, when migration is not possible, *D. haemobaphes* is capable of withstanding the direct co-existence with *D. villosus* without any visible negative consequences in shelter use, at least over a short term tested in our study.

In accordance with our hypothesis 5, infection status did affect interspecific interactions among amphipods. Infected and non-infected amphipods did not differ from each other in their shelter occupancy time in the presence of heterospecific opponents, but the infection status of the opponent did affect the responses of *D. haemobaphes*: they spent more time in the shelter in the presence of infected rather than non-infected heterospecifics (Fig. 3A). Thus, similarly to intraspecific interactions, they utilized shelters more efficiently in the presence of infected *D. villosus*. It has been already evidenced that the presence of microsporidian and/or acanthocephalan parasites reduces the intraguild predation pressure among amphipod species and facilitates their coexistence (e.g. MacNeil and Dick 2011).

In contrast to intraspecific interactions, amphipods did not change their aggression rate depending on the infection status of the accompanying heterospecific. Perhaps they are only able to recognize the infection in conspecific competitors, or the level of interspecific aggression is already so low that the danger of getting infected after biting an infected heterospecific competitor is negligible.

To summarize, according to our hypothesis 5, infection increased amphipod shelter occupancy in heterospecific dyads and thus could contribute to the co-existence of the two species over a longer time scale. Nevertheless, the effect of infection on interspecific relationships was less pronounced, particularly in terms of aggression changes, than in the case of intraspecific interactions.

Conclusions

In general, parasites tended to reduce the ability of their hosts to defend their shelters, though did not directly reduce their aggression. This indicates the reduced competitive abilities of infected amphipods and relatively improved performance of their non-infected opponents. However, in terms of shelter occupancy time, overall benefits of the non-infected individuals seem greater than losses of the infected animals, particularly given the fact that amphipods tended to reduce their aggression towards infected conspecifics. *Dikerogammarus haemobaphes* benefited (in terms of the longer shelter occupancy) from the presence of infected conspecifics and heterospecifics, whereas *D. villosus* increased its shelter occupancy only in response to infected conspecifics. Thus, parasites, apart from their apparent negative direct effects on their hosts, at the population and community levels may promote species co-existence rather than displacement. Obviously, confirmation of such a conclusion needs a longer-term study than our 30-min long experiment, but shelter use is an important life parameter of these sit-and-wait organisms, shaping their performance in the wild to a high extent. Although the Microsporidium species under our study causes a lethal disease, its presence may temporarily, before the terminal phase, result in locally increased population densities due to the lower levels of interference competition. This, in turn, may increase the impact of the amphipod assemblage on the local community. Given highly variable (both spatially and temporally) levels of Microsporidium prevalence in amphipod assemblages (Bącela-Spychalska et al. 2012), parasite presence may account for contrasting outcomes of their interspecific competition, leading to displacement or coexistence. If *C. dikerogammari* infection had been more virulent to *D. villosus* (a theoretically stronger competitor) than to *D. haemobaphes*, the parasite would have been likely to sustain their coexistence through apparent competition. Whether these mechanisms translate into cascading ecosystem level effects on other organisms, such as local predators, prey and competitors of invasive amphipod assemblages, remains to be determined in future studies. Nevertheless, parasitic infection seems to be a likely and so far overlooked factor contributing to discrepancies among various studies describing the effects of the Ponto-Caspian community on invaded communities. For

instance, *D. villosus* has been shown as an efficient carnivore (Dick et al. 2002; Krisp and Maier 2005; Bącela-Spychalska and Van der Velde 2013), occupying the niche corresponding to that of small fish (van Riel et al. 2006) and showing low activity as a shredder of coarse plant detritus (MacNeil et al. 2011), but also, in contrast to the above-mentioned statements, as an efficient shredder and herbivore with a low share of food of animal origin in its diet (Hellmann et al. 2015; Koester et al. 2016).

Our study shows that parasitic infections play an important role in shaping biological invasions not only by mediating interactions between invasive and local organisms, as it has been shown previously (Dunn 2009; Dunn et al. 2012; Dunn and Hatcher 2015; Hatcher et al. 2015), but also by affecting interactions among various invasive species and likely shaping their impact on the environment. The presence of Microsporidia in our model system may contribute to the invasional meltdown phenomenon (Simberloff and Von Holle 1999) by improving the coexistence of invasive species. Incorporating additional invasive species, in this case the parasites, makes the composition of the invasive host species assemblage more complex. Without microsporidians infecting the top competitor, the weaker species could be displaced, but, in the presence of these parasites, it may be able to face the infected opponent. As a result, the number of invasive species in the community (consisting of hosts and parasites) may be expected to be higher than in a parasite-free community. On the other hand, microsporidian parasites infecting *D. villosus* may diminish its predatory pressure and moderate its impact on local biota (as shown by Bącela-Spychalska et al. 2014), thus reducing its negative impact. This would be in contrast to another assumption of the invasional meltdown hypothesis, namely the exacerbated impact of invasive species on local biota due to the appearance of additional invaders (Simberloff and Von Holle 1999). Thus, the addition of alien parasites to the community may have contradictory effects on the appearance of the invasional meltdown phenomenon.

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Supplementary material 1

Figure S1, Tables S1, S2

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Data type: Pdf file

Explanation note: Figure S1. Experimental setup. Table S1. Numbers of particular amphipod pairs obtained in the study. Table S2. Analyses carried out within the study.

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Supplementary material 2

Dataset

Authors: Jarosław Kobak, Michał Rachalewski, Karolina Bącela-Spychalska

Data type: excel file

Explanation note: Experimental data.

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